How to be good at being a virus

Berngruber, Thomas

Chapter 4
(Addition to chapter 3) Is the fitness loss of phage ΦX174 caused by competition with a pro-phage?

[1]Thomas Berngruber, [2]Olivier Tenaillon and [1]Franz J. Weissing

[1]Theoretical Biology, Centre for Ecological and Evolutionary Studies, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands . [2]INSERM EMI0339, Faculte Xavier Bichat, 16 rue Henri Huchard 75870 Paris cedex 18, France,

4.1 Abstract

In a previous experiment several lines of phage ΦX174 that were adapted to a Petri-dish environment were transferred to a liquid environment were they could evolve for 130 transfers in batch culture. Unexpectedly viral fitness decreased (rather than increased) in the novel environment. This finding might be explained by competition of ΦX174 with a different species of phage. Here we show that the experimental lines did indeed contain a previously unknown phage. This phage has most likely entered our experiments from a pro-phage stage that is integrated in the genome of the host bacterium E.coli C122 mutT. We sequenced fragments of the genome of the unknown phage and compared them to known phage sequences. It turned out that the

unknown phage is a close relative to the lysogenic phages N15, HK22 and Φ80. We discuss possible mechanisms by which this unknown phage could reduce the reproduction of ΦX174.

4.2 Introduction

In a previous experiment several lines of phage ΦX174 that were adapted to a Petri-dish environment were transferred to a liquid environment were they could evolve for 130 transfers in batch culture. Unexpectedly viral fitness decreased (rather than increased) in the novel environment. This observation was puzzling since adaptation to a constant environment should generally lead to an improvement of reproductive output. After excluding alternative explanations we arrived at the conclusion that the fitness decrease of ΦX174 is caused by competition with a second species of phage. In order to detect and characterize this unknown phage we isolated viral DNA from a culture of E.coli C122 mutT, sequenced fragments of the viral DNA and compared them to known phage sequences.

4.3 Materials & Methods

E.coli C122 mutT was grown in LB (10 mM MgCl2 and 5 mM CaCl2) for 3h at 37°C until early stationary phase. To induce the unknown lysogenic phage, the culture was UV irradiated in a Petridish with 4.5 W, 233 nm UV (Mineralight UVS-54 handheld UV lamp) for 15 min and diluted 1:2 with LB medium and grown for one hour. The culture was filtered by 0.2 µm cellulose acetate filter (*Millipore*) and incubated with 200 U/ml DNAse (*Roche*) for 30 min at room temperature in order to digest remaining host genomic DNA. The DNAse activity was stopped by 50 mM EDTA, prior to destruction of the phage coat by 1% SDS and 5 vol% of 1:1 phenol/chloroform. The phage DNA was precipitated by 3 volumes of cold 100% Et-OH and 1 volume 3M Na-acetate. The isolated DNA was run on a 0.8% agarose electrophoresis gel. Phage DNA for cloning was digested by EcoRI and HindIII (*New England Biolabs*) overnight. The resulting restriction fragments were

ligated into EcoRI and HindIII digested plasmid puc18. Plasmids that carried an insert of phage DNA were sequenced by standad lacZ primers (*GATC Biotech AG*). The resulting sequences were queried against known sequences using BLAST (*NCBI*).

4.4 Results

DNA isolation and electrophoresis from the liquid fraction of an UV induced culture of E.coli C122 mutT showed a band of approximately 30kb which might represent the unknown phage (Figure 1). Sub-cloning and partial sequencing of 4 of the cloned DNA insertions revealed high sequence similarity with regions from the lysogenic phages Φ80, HK22 and N15 (see Table 1). The unknown phage is possibly a chimera of these lysogenic phages. Formation of chimeric variants between these lysogenic phages is common (Juhala et al. 2000). Yet, the presence of multiple lysogenic phages of similar genome size cannot be excluded.

Table 1: Sequence similarity of the unknown lysogenic phage with known phage sequences

Match	***Position***	***Nucleotide Similarity***
Accession number		
Bacteriophage Φ80	*2-126 (major coat*	*125 out of 125 (100%)*
emb|X13065.1|BP80ER	*protein)*	*560 out of 567 (98%)*
	5447-6013 (early region)	
Bacteriophage N15	*5479-6143*	*634 out of 665 (95%)*
gb|AF064539.1|AF064539		
Bacteriophage HK022	*17218-17490*	*250 out of 273 (91%)*
gb|AF069308.1|AF069308		

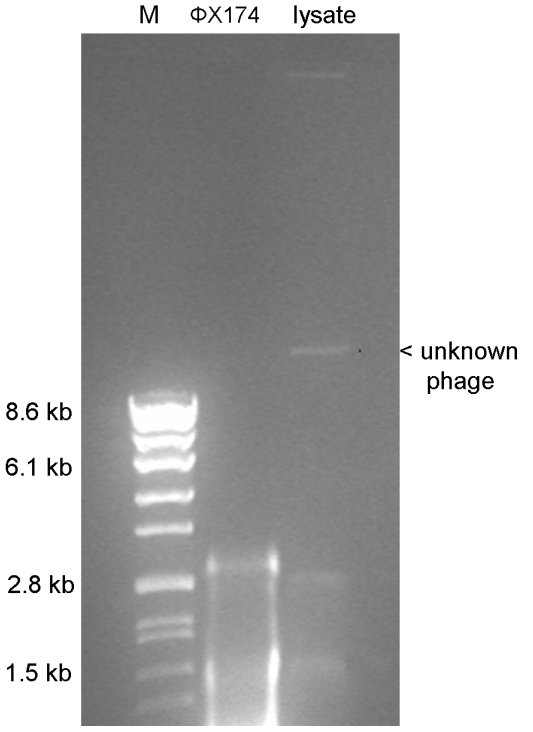

Figure 1: DNA isolation of phage ΦX174 and a UV induced lysate of host E.coli C122 mutT. First lane (M) contains marker DNA. Second lane (ΦX174) contains DNA from a ΦX174 isolate. Third lane (lysate) contains DNA of the unknown lysogenic phage that was extracted from the liquid fraction of an *E.coli C122 mutT* culture after UV induction. The genome size of the unknown phage is approximately 30 kb. [Note: The DNA band of ΦX174 appears smaller than its actual genome size (5.1 kb) as unrestricted ΦX174 DNA appears in its super-coiled form which runs faster in electrophoresis than linear DNA of the same size]

4.5 Discussion

We identified a previously unknown lysogenic phage which might be responsible for the decrease in reproductive efficiency of the lytic phage ΦX174 in our earlier experiments. This contaminant phage turned out to be notoriously difficult to detect and had therefore remained undetected for years, also in the work of several other labs. Lysogenic phages are known to stably integrate into the genome of the host bacterium and propagate to bacterial daughter cells through vertical transmission. We assume that the unknown lysogenic phage has entered our experiment from a pro-phage stage in the host bacterium *E.coli C122 mutT*, since the preperation of our host strain (loose colonies on an agar plate) exclude horizontal transmission.

Lysogenic phages are common. In a survey from the year 2003, 51 out of 82 full bacterial genome sequences were shown to contain at least one pro-phage, while some genomes contained up to 17 pro-phages accounting for approximately 10% of the bacterial genome (Casjens 2003). Even though lysogenic phages are common, they can easily go unnoticed. Lysogenic phage which are stably integrated into the host genome can have little effect on the host phenotype until a lytic cycle is artificially induced (e.g. by UV-mutagenesis or Mytomycin) (Oppenheim et al. 2005). Furthermore, classical plaque formation assays do often not detect lysogenic phage particles, since the original pro-phage carrying host strain is immune to infection of phage particles of the same species (Susskind et al. 1974; Kliem & Dreiseikelmann 1989). Phage particles therefore generally do not form plaques on their original carrier host. Our unknown lysogenic phage for example does not form plaques on its original host E.coli C122 mutT, but does form plaques on other pro-phage free E.coli strains. Detection of lysogenic phage therefore requires serendipity like the accidental plating on a pro-phage free host strain or a spontaneous lysis event. Alternative methods for the detection of lysogenic phages like electron microscopy or sequencing are quite involved. In our experiments the first indication

for the occurrence of the lysogenic phage was therefore the reduction of the fecundity of ΦX174 through competition with the lysogenic phage.

Even though the described interaction between a lysogenic and a lytic phage is accidental, it raises important question on (1) the mechanisms that trigged the activation of the lysogenic phage from the pro-phage state; (2) the mechanisms of competition by which the lysogenic phage reduces the reproduction of ΦX174; and (3) the selection on phage life-history that results from the competition of a lytic and a lysogenic phage in general. Competition between different species of viruses in mixed infections can result in significant loss of fecundity for both competitors (Huppert et al. 1967; Hattman & Hofschne 1967; Freda & Buck 1971). Yet, the mechanisms of competition are often unknown. In general the competition between two different species of viruses can occur on three levels: Competition for the availability of host cells, competition for resources during co-infection and the prevention of co-infection by interference mechanisms.

The ability to compete for available host cells is important for the success of a virus in our experimental treatment and is prone to select for altered life histories of both competitors. In every transfer we add a phage lysate that contains both competitors to a naïve host population (that carries the pro-phage). Rapid infection of the host population is therefore decisive for viral competition. A phage can increase its rate of infection by increasing its density in the lysate or by increasing its infectivity. Competition for available host cells therefore creates selective on two viral life-history parameters. First, competition for host cells selects for increased release of phage particles resulting in increased horizontal transmission. Second, competition for available host cells selects for increased rates of infection. Competition for host cells should therefore select for increased rates of infection in both phage and for an increased rate of horizontal transmission of the lysogenic phage. The selection for increased rates of infection might give an alternative explanation for the increase in adsorption rate of

ΦX174 that we observed in the experiments in chapter 3. Furthermore, the selection for increased horizontal transmission of the lysogenic phage can be a possible mechanism by which the lysogenic phage progressively increases its representation in the phage lysate and this way reduces the reproduction of ΦX174.

Alternatively, the competition for resources between phages that co-infect one host cell can be another mechanism by which the unknown lysogenic phage reduces the reproductive output of ΦX174. Within-host competition during co-infection acts though competition for the transcription and translation machinery of the host cell. Infection of the lytic DNA phage T4 , for example, can reduce the reproduction of RNA phage by competition for viral protein production (Hattman & Hofschne 1967) through competition of messenger RNAs (Goldman & Lodish 1975). Even though, intra-cellular competition for resources has not been studied in mixed infections with lysogenic phage, the competition for the production of proteins and messenger RNA are fundamental intra-cellular trade-offs that should also apply for competition of ΦX174 and the unknown lysogenic phage.

Next to competition for resources of lysogenic and lytic phage can involve interference mechanisms. In the lysogenic phage λ, for example, the exclusion proteins rexA and rexB prematurely lyse the host cell when a co-infecting lytic phage is detected and this way abolish the reproduction of the co-infecting competitor (Parma et al. 1992). The mechanism of premature cell lysis predicts a shortening in lysis timing. In contrast to this prediction we observed an increase in the burst time of all evolved strains (chapter 3, Figure 5). Premature lysis by the action of rexA and rexB is therefore not likely to be responsible for the reduced reproductive output of ΦX174.

4.6 References

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Chapter 5
Viral superinfection inhibition and the evolution of virulence

Thomas W. Berngruber and Franz J. Weissing

Theoretical Biology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, The Netherlands

5.1 Abstract

The competition of multiple pathogen strains within a single host is strongly affected by the replication rate and virulence of the competing strains. Strains with a higher replication rate and, hence, a higher virulence will typically have a competitive advantage. For this reason, many models for the evolution of virulence under superinfection assume that more virulent pathogens are better protected against superinfection. However this expectation is reversed in a broad range of benign viral systems, which deploy molecular superinfection inhibition mechanisms in order to win within-host competition. Superinfection inhibition mechanisms can lead to an increased rate of superinfection with increased virulence of the first infecting virus. By means of an evolutionary model that includes the molecular mechanisms of superinfection inhibition of bacteriophage λ we show that superinfection inhibition can lead to novel evolutionary dynamics like the evolutionary coexistence of virulent and non-virulent strains. Molecular mechanisms of superinfection inhibition also occur in other

benign viruses of different origins like phage M13, Hepatitis B Virus or Foamy Virus. Neglecting the mechanisms can lead to erroneous predictions on the outcome of viral evolution. In fact, these mechanisms might be crucial for the maintenance of viral benignity.

5.2 Introduction

The genomes of cellular organisms are interspersed by large numbers of viral genomes that reside in a dormant state. For example, 8% of the human genome consists of sequences of retroviral origin (Lander et al. 2001). Likewise, the average number of dormant pro-phage in all sequenced bacterial genomes is 2.6 and some bacterial genomes contain up to seventeen dormant viruses that constitute 10% of their total genome (Casjens 2003). It is still largely unknown why that many viruses remain dormant, while a more virulent strategy seems to provide obvious fitness benefits.

The evolution of virulence is affected by between-host and within-host competition. Between-host competition may reduce virulence when the reduction of host density, caused by virulence, hampers transmission (Anderson and May 1982; Ewald 1983). In contrast within-host competition is generally thought to favor increased virulence since rapidly replicating virulent strains have a competitive advantage over more slowly replicating variants (May and Nowak 1995; Nowak and May 1994; van Baalen and Sabelis 1995b; Frank 1996; Gandon et al. 2001; de Roode et al. 2005). The relative importance of within-host and between-host processes is still under debate. It has been argued (e.g. Ebert and Bull 2003) that direct within-host competition should typically dominate over between-host competition that is mediated by more indirect processes like the reduction of host density. If this is indeed the case an explanation has to be given how within-host competition can lead to reduced virulence and even viral dormancy.

The prediction that within-host processes should generally select for increased virulence are usually based on simple conceptual models that

do not consider mechanistic details of the competitive interactions of viral strains during co-infection. Yet, such details may be of crucial importance. An example are defective interfering particles (DIPs) that parasitize on the protein production of wildtype virus (Turner and Chao 1999; Chao et al. 2000; Dennehy and Turner 2004). By exploiting the wildtype, DIPs can spread, thereby reducing the overall production of viable viruses and, hence, virulence. However, DIPs can never spread to fixation, since they remain dependent on a co-infecting virulent wild-type strain. Accordingly, DIPs can partially explain a reduction of viral virulence, but not the widespread evolution of viral dormancy.

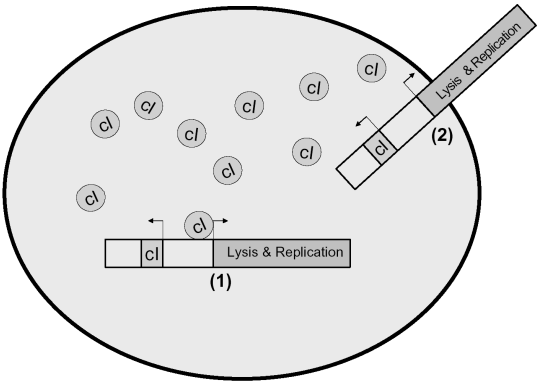
Here we discuss another mechanism that can yield such an explanation. In order to protect themselves from within-host competition, many RNA and DNA viruses of bacteria, plants and animals have evolved so-called superinfection inhibition that prevents the infection of already infected cells by other viral strains of the same viral species (Hutchison and Sinsheimer 1971; Susskind et al. 1974; Mcallister and Barrett 1977; Kliem and Dreiseikelmann 1989; Christen et al. 1990; Simon et al. 1990; Karpf et al. 1997; Ellenberg et al. 2004; Lee et al. 2005; Nethe et al. 2005; Huang et al. 2008). The mechanisms underlying viral superinfection inhibition are well studied. They often involve the repression of the replication machinery of the superinfecting virus. In many cases the molecular mechanisms that repress a co-infecting strain also limit self-replication of the resident virus and, accordingly, its virulence. Hence, reduced virulence might be a side effect of superinfection inhibition.

The mechanisms underlying the trade-off between superinfection inhibition and virulence are well studied in bacteriophage λ (Figure 1) (Bailone and Devoret 1978; Susskind and Youderain 1983; Oppenheim et al. 2005) and other benign viruses (Kliem and Dreiseikelmann 1989; Christen et al. 1990; Simon et al. 1990; Ellenberg et al. 2004; Lee et al. 2005; Nethe et al. 2005). Phage λ is a temperate bacterial virus that can propagate vertically, integrated as a pro-phage into the genome of its

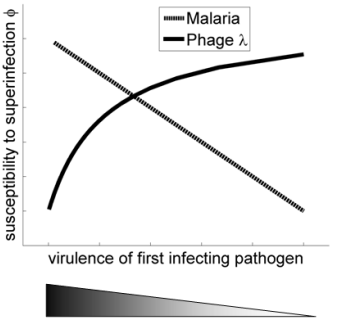
host, or horizontally by initiation of replication and host lysis. Since horizontal transmission requires host lysis it inevitably causes virulence. In contrast, during vertical transmission the pro-phage maintains a stable dormant state and virulence is low. The genetic mechanisms that repress the switch to the lytic cycle therefore control virulence. In phage λ, this switch to the lytic cycle is achieved by binding of the virulence repressor cI to the $p_L p_R$ promoter that controls the viral lysis and replication genes (Johnson et al. 1981; Ptashne 2004; Oppenheim et al. 2005). By the same mechanism the cI repressor also provides the host cell with superinfection inhibition against a second phage, when the cI repressor binds to the $p_L p_R$ promoter of the superinfecting phage (Figure 1A). Yet, this inhibition can be avoided by mutations in the the $p_L p_R$ promoter of the superinfecting phage (Figure 1A). These mutants however lose the ability to control their own virulence and are therefore termed ultra-virulent (Bailone and Devoret 1978).

Mechanisms as the one described above have not yet been incorporated into models of pathogen evolution. Generally superinfection models assume a decrease in the susceptibility to superinfection with increasing virulence of the resident pathogen (Mosquera and Adler 1998; Pugliese 2002; Boldin and Diekmann 2008), as has been demonstrated for malaria pathogens (de Roode et al. 2005) (Figure 1B). As the example of phage λ and other viral pathogens shows, it is by no means clear whether, and to what extent, this assumption is generally valid. It is therefore not self-evident that within-host competition should generally lead to higher virulence.

(A)



(B)



<u>Figure1: Virulence regulation and super-infection inhibition in phage λ</u> (A) A resident phage stays in the lysogenic state when the virulence repressor cI is bound to its pLpR promoter (1), thereby suppressing the resident's lysis and replication genes. By the same mechanism cI binds to the pLpR promoter of a superinfecting phage (2), thereby preventing its replication. This inhibits superinfection. Hence, viruses that produce a low level of repressor cI are more virulent and at the same time more susceptible to superinfection. (B) The mechanisms above create a positive association between virulence of the resident phage and the susceptibility to superinfection. This is in contrast to pathogens like malaria parasites where more virulent strains are less susceptible to superinfection.

In order to study the effect of these mechanisms on the evolution of viral virulence, we developed an evolutionary model that integrates biochemical mechanisms into a population model which considers the invasion of a rare viral mutant into an established ecological equilibrium of a resident virus. By this approach we aim to integrate the biochemical, ecological and evolutionary scales of superinfection and to provide a mechanistic view on the evolution of viral virulence under superinfection inhibition.

5.3 A model for the evolution of virulence under superinfection inhibition

We consider a virus that spreads by vertical and horizontal transmission between individual host cells. For simplicity we assume that the virus is highly infective and that, correspondingly, virtually all host cells are infected. Therefore a viral mutant can only increase in frequency when it is able to superinfect a host cell that is already infected by a resident virus. We will focus on the situation where co-infection eventually leads to take-over of the host cell by one of the competitors. The competition between the resident and a mutant strain of a virus can then be studied by following the dynamics of host cells that are infected by either type of virus (Nowak and May 1994). We represent this situation by two coupled differential equations that describe the density of hosts infected by the resident virus y_R and hosts infected by a mutant y_M, respectively:

$$\frac{dy_R}{dt} = ry_R\left(1-y_R-y_M\right)-\alpha_R y_R+\beta\left(\alpha_R\Phi_{MR}-\alpha_M\Phi_{RM}\right)y_R y_M \qquad (1a)$$

$$\frac{dy_M}{dt} = ry_M\left(1-y_R-y_M\right)-\alpha_M y_M+\beta\left(\alpha_M\Phi_{RM}-\alpha_R\Phi_{MR}\right)y_R y_M \qquad (1b)$$

The system has the following interpretation. In the absence of viral infection, the host grows logistically with intrinsic growth rate r and a carrying capacity that is normalized to 1. This part of the growth equation includes all mortality not induced by the viruses and it

determines the rate of vertical transmission of the viruses. The terms $\alpha_R y_R$ and $\alpha_M y_M$ correspond to virus induced mortality. Hence α_R and α_M characterize the virulence of resident and mutant, respectively. Virus-induced mortality is associated with cell lysis and viral reproduction. The number of newly produced resident virus is therefore proportional to $\alpha_R y_R$. The rate at which these viruses attempt to superinfect host cells incorporating the mutant virus is proportional to their abundance $\alpha_R y_R$ and to the abundance y_M of mutant-infected hosts. The constant of proportionality β includes the yield of virus production, diffusivity of the medium and the adsorption rate upon encounter with a host cell (which we assume to be the same for both types of virus). The crucial ingredient in our model is the superinfection term Φ_{MR}, which corresponds to the proportion of superinfection attempts that result in a 'take-over' of a mutant-infected host by the resident virus. Hence the rate of recruitment of new resident infected hosts due to successful superinfection by resident viruses is given by $\beta\alpha_R y_R y_M \Phi_{MR}$. Similarly, the recruitment of new mutant-infected hosts due to successful superinfection by mutant viruses is given by $\beta\alpha_M y_M y_R \Phi_{RM}$. Notice that the indices in the superinfection terms Φ_{RM} and Φ_{MR} reflect the order of arrival of the two types of viruses. Hence, Φ_{AB} corresponds to the probability that upon adsorption to a host infected by A a newly formed virus B takes over this host (Mosquera and Adler 1998).

When we consider the resident virus in the absence of a mutant (i.e. $y_M = 0$), the resident host population reaches the population dynamical equilibrium

$$y_R^* = \frac{r - \alpha_R}{r} \tag{2}$$

In this resident equilibrium a rare mutant can increase in frequency when its per capita growth rate is positive:

$$\frac{1}{y_M}\frac{dy_M}{dt}\bigg|_{y_R=y_R^*,\,y_M=0} = \alpha_R - \alpha_M + \beta\frac{r-\alpha_R}{r}\left(\alpha_M\Phi_{RM} - \alpha_R\Phi_{MR}\right) > 0\,. \qquad (3)$$

The per capita growth rate of a mutant in the resident equilibrium corresponds to the invasion fitness of the mutant (Geritz et al. 1998) that we will denote by W. Equation (3) shows how invasion fitness depends on virulence α and the susceptibility to superinfection Φ of both, the resident and the mutant virus. Although the rate of superinfection is often directly described as a function of virulence, e.g. $\Phi_{RM}(\alpha_R,\alpha_M)$, it is likely that virulence α and the rate of superinfection Φ are more indirectly related, e.g. via a correlation to some underlying trait or the concentration of some protein (like the virulence repressor protein cI of phage λ). In order to allow for both possibilities we choose a general approach in which α and Φ are functions of some trait x (that we will specify later). In other words we assume that

$$\alpha_R = \alpha(x_R),\ \Phi_{RM} = \phi(x_R, x_M) \qquad (4a)$$

and

$$\alpha_M = \alpha(x_M),\ \ \Phi_{MR} = \phi(x_M, x_R) \qquad (4b)$$

where x_R and x_M are the trait value of the resident and the mutant virus, respectively. Now, the invasion fitness can be rewritten as

$$W(x_M,x_R) = \alpha(x_R) - \alpha(x_M) + \beta\frac{r-\alpha(x_R)}{r}\left[\alpha(x_M)\phi(x_R,x_M) - \alpha(x_R)\phi(x_M,x_R)\right] \qquad (5)$$

Obviously $W(x_R, x_R) = 0$. This makes sense, since the resident should neither grow nor decline in a population of residents. A rare mutant with trait x_M will invade when $W(x_M, x_R) > 0$. The direction of selection (i.e. whether selection favors larger or smaller values of x) is therefore given by the selection gradient

$$\left.\frac{\partial W}{\partial x_M}\right|_{x_M=x_R} = -\alpha'(x_R) + \beta\frac{r-\alpha(x_R)}{r}\left(\alpha'(x_R)\phi(x_R,x_R) - \alpha(x_R)\left(\left.\frac{\partial\phi(x_M,x_R)}{\partial x_M} - \frac{\partial\phi(x_R,x_M)}{\partial x_R}\right|_{x_M=x_R}\right)\right) \quad (6)$$

Of particular importance are those resident strategies x_R^* where there is no directional selection any more (so-called evolutionarily singular strategies; Geritz et al. 1998), i.e. those resident strategies $x_R = x_R^*$ for which

$$\left.\frac{\partial W}{\partial x_M}\right|_{x_M = x_R^*} = 0 \quad (7)$$

The resident strategy x_R^* is an evolutionarily stable strategy (ESS) when the invasion fitness $W(x_M, x_R^*)$ has a maximum in the direction of the mutant strategy x_M or

$$\left.\frac{\partial W}{\partial x_M}\right|_{x_M = x_R^*} = 0 \quad \text{and} \quad \left.\frac{\partial^2 W}{\partial x_M^2}\right|_{x_M = x_R^*} < 0. \quad (8)$$

At an ESS all mutant traits x_M in the vicinity of x_R^* have a lower fitness than the resident. Accordingly, a resident population with strategy x_R^* is immune by invasion against mutants. An ESS is not necessarily reachable by a series of small gene substitution events

(Geritz et al. 1998). The resident strategy x_R^* is convergence stable (i.e. an evolutionary attractor) if

$$\left.\frac{\partial W}{\partial x_M}\right|_{x_M=x_R^*} = 0 \quad \text{and} \quad \left.\frac{\partial}{\partial x_R}\left[\left.\frac{\partial W}{\partial x_M}\right|_{x_M=x_R^*}\right]\right|_{x_M=x_R^*} < 0. \tag{9}$$

When x_R^* is convergence stable but not evolutionarily stable, evolution will converge to an evolutionarily unstable strategy; a so-called branching point. In this case, evolution a polymorphism of two (or more) coexisting strategies will result.

5.4 Simplified scenarios

5.4.1 Scenario 1: Superinfection directly related to virulence

We will now show that the relation between virulence and susceptibility to superinfection is decisive for the dynamics and outcome of evolution. To this end, we first consider the special case where virulence itself is the target of selection, i.e.

$$x_R = \alpha_R \text{ and } x_M = \alpha_M \tag{10}$$

Moreover we make the simplifying assumption that the rate of superinfection only depends on the virulence of the first infecting phage, which is related to its repressor concentration, or

$$\Phi_{RM} = \phi(\alpha_R),\ \Phi_{MR} = \phi(\alpha_M) \tag{11}$$

Thereby ϕ is a function taking on values between 0 and 1 and characterizes the nature of the trade-off between virulence of the first infecting phage and susceptibility to superinfection. (We will elucidate the biochemical link between repressor concentration, virulence and

superinfection in the next section.) Under these assumptions (10) and (11), the invasion fitness is given by

$$W(\alpha_M,\alpha_R) = \alpha_R - \alpha_M + \beta \frac{r-\alpha_R}{r} \left[\alpha_M \phi(\alpha_R) - \alpha_R \phi(\alpha_M)\right] \tag{12}$$

An evolutionarily singular strategy α_R^* is now given by the condition

$$\left.\frac{\partial W}{\partial \alpha_M}\right|_{\alpha_M=\alpha_R^*} = -1 + \beta \frac{r-\alpha_R^*}{r} \left[\phi(\alpha_R^*) - \alpha_R^* \phi'(\alpha_R^*)\right] = 0 \tag{13}$$

or equivalently

$$\beta \left[\phi(\alpha_R^*) - \alpha_R^* \phi'(\alpha_R^*)\right] = \frac{r}{r-\alpha_R^*} \tag{14}$$

In view of

$$\left.\frac{\partial^2 W}{\partial \alpha_M^{\,2}}\right|_{\alpha_M=\alpha_R^*} = -\beta \frac{r-\alpha_R^*}{r} \alpha_R^* \phi''(\alpha_R^*) \tag{15}$$

a positive solution $\alpha_R^* > 0$ of (14) is evolutionarily stable whenever $\phi''(\alpha_R^*) > 0$, or in other words whenever the superinfection function is convex in the vicinity of α_R^*. Making use of (14), the condition for convergence stability is given by

$$\left.\frac{\partial}{\partial \alpha_R}\left[\left.\frac{\partial W}{\partial \alpha_M}\right|_{\alpha_M=\alpha_R^*}\right]\right|_{\alpha_M=\alpha_R^*} = -\frac{r}{r-\alpha_R^*} - \beta \frac{r-\alpha_R^*}{r} \alpha_R^* \phi''(\alpha_R^*) < 0 \tag{16}$$

This is automatically satisfied whenever $\phi''(\alpha_R^*) > 0$. In other words, and evolutionarily stable strategy is always convergence stable.

However, an evolutionarily unstable strategy can also be convergence stable if $\phi''\left(\alpha_R^*\right)$ is not too negative. To be precise, a solution of (14) is a branching point whenever

$$-\frac{r}{\beta\alpha_R^*\left(r-\alpha_R^*\right)^2} < \phi''\left(\alpha_R^*\right) < 0 \tag{17}$$

Numerical analysis through a pairwise invasibility plot shows that in this case virulence α_R^* either evolves towards the branching point or towards the non-virulent strategy $\alpha_R^* = 0$, depending on the initial level of virulence (Figure 2).

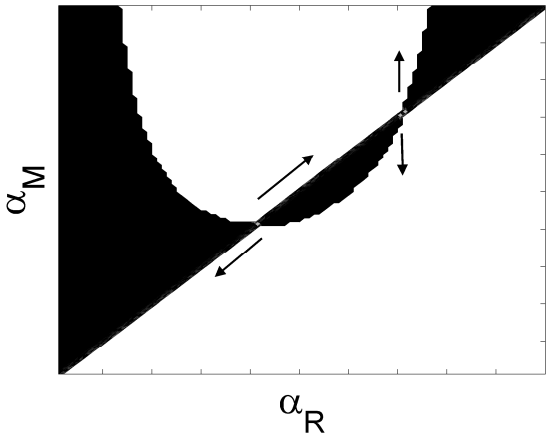


Figure 2: Pair wise invasibility plot for a concave superinfection function. A concave relation between the susceptibility to superinfection and virulence of the first infecting strain ($\phi''(\alpha) < 0$) can lead to three evolutionarily singular points: The ESS $\alpha^* = 0$, an evolutionary branching point for virulence and an evolutionary repellor which separates the two strategies (Superinfection function: $\phi(\alpha) = \alpha/(\alpha+1)$ Parameters: $\beta = 20$, $r = 1$).

5.4.2 Scenario 2: Virulence and susceptibility to superinfection are determined by repressor binding

In the previous section we showed that a concave positive relation between the rate of superinfection and the virulence of the first infecting virus can lead to evolutionary branching of virulence or the maintenance of a non-virulent population. Yet, we did not elucidate the origins of the relation between the susceptibility to superinfection and virulence. Therefore, in this section, we will derive this relation from the molecular mechanisms of viral virulence and superinfection control, along the example of bacteriophage λ. In λ the virulence α and the rate of superinfection ϕ are both determined by sigmiodal binding dynamics of the repressor cI to the pLpR promoter (Johnson et al. 1981; Hendrix et al. 1983; Ptashne 2004). Following these binding dynamics the virulence α_R of the resident phage is determined by the resident repressor concentration c_R and the resident promoter affinity k_R. The rate of superinfection ϕ_{RM} is in turn determined by the repressor concentration of the resident phage c_R and the promoter affinity of the super-infecting phage k_M (see Figure 1A). This way we can describe the proportion p of host cells, which have a repressor molecule bound to the $p_L p_R$ promoter, and remain in the lysogenic state, by second order receptor binding kinetics in the form

$$p = \frac{c^2}{k^2 + c^2} \tag{18}$$

In turn, the proportion of cells that switch to the lytic cycle is $1 - p$. Since the switching rate to the lytic cycle is equivalent to virulence α, we can write

$$\alpha_R\left(c_R, k_R\right) = 1 - p_R = \frac{{k_R}^2}{{k_R}^2 + {c_R}^2}, \tag{19a}$$

and

$$\alpha_M\left(c_M,k_M\right)=1-p_M=\frac{{k_M}^2}{{k_M}^2+{c_M}^2}. \qquad (19b)$$

On the other hand the susceptibility to superinfection is determined by the repressor concentration of the resident phage c and the promoter affinity of the super-infecting phage k, or

$$\phi_{RM}\left(c_R,k_M\right)=\gamma\frac{{k_M}^2}{{k_M}^2+{c_R}^2} \qquad (19c)$$

and

$$\phi_{MR}\left(c_M,k_R\right)=\gamma\frac{{k_R}^2}{{k_R}^2+{c_M}^2} \qquad (19d)$$

where γ is the relative binding efficiency between self and foreign promoter binding.

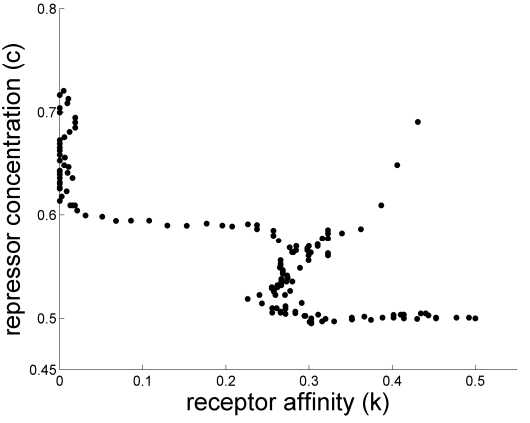
By the use of $\alpha_R\left(c_R,k_R\right)$, $\alpha_M\left(c_M,k_M\right)$, $\phi_{RM}\left(c_R,k_M\right)$ and $\phi_{MR}\left(c_M,k_R\right)$ we can now rewrite the system of two competing strains (1a, 1b) in terms of the underlying biochemical properties. This way we can represent the two trade-offs between virulence and superinfection inhibition, and the avoidance of superinfection inhibition and virulence, directly by the biochemical properties c and k. We can follow the evolution of the biochemical properties c and k by the numerical method of an evolutionary walk. Thereby, we introduce a rare mutant of c_M or k_M into a resident population c_R and k_R and integrate the system (1a, 1b) given the relations (8, 9). When a mutant increases in frequency and displaces the resident, the mutant becomes the new resident and the evolutionary path makes one step. By iteration of this process we can follow the correlated evolution of c and k and its effect on virulence α and the rate of superinfection ϕ.

The evolution of virulence for the biochemical model (1a, 1b, 8, 9) leads to evolutionary branching in virulence α, but evolution in the underlying parameters now proceeds in two dimensions (Figure 3). This leads to an adaptation process in several steps. At first virulence α decreases through directional selection towards lower receptor affinity k until it approaches the branching point. Close to the branching point the repressor concentration c increases until the population divides into two strategies: Low receptor affinity and low virulence and high receptor affinity and high virulence (Figure 3A). This way a single ancestor strategy can evolve into two coexisting strategies: A defense specialist that is analogous to the lysogenic phage λ wildtype and an attack specialist that is analogous to ultra-virulent mutants of phage λ. This example provides a mechanism by which virulent and non-virulent viruses can stably coexist in a natural virus population.

5.5 Disccussion

Within-host competition between parasites strains has fundamental consequences for the evolution of pathogen virulence (Pugliese 2002; Boldin and Diekmann 2008; de Roode et al. 2005; Nowak and May 1994; Gandon et al. 2001; Adler and Mosquera 2000). The competitive interactions of pathogen strains during co-infection are complex. A common approach to simplify these competitive interactions is the introduction of a superinfection function which describes the rate at which a first infecting pathogen is out competed and replaced by a second pathogen from its host cell. The superinfection function can be interpreted as a limiting case of co-infection with an instantaneous replacement of pathogens through competition.

(A)



(B)

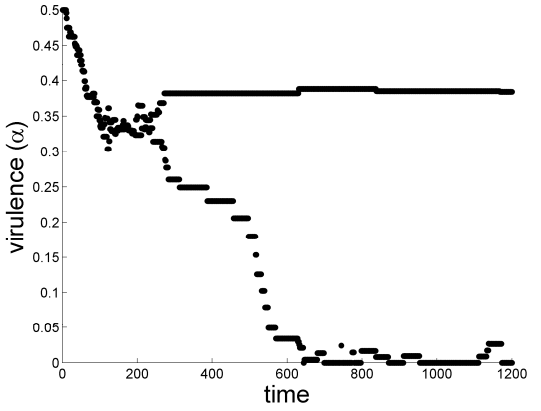


Figure 3: Evolutionary branching of virulence reflects the biochemical details of phage λ repressor binding. (A) Correlated evolution of repressor concentration and receptor affinity determine the evolution of virulence. Selection towards lower virulence decreases receptor affinity (= stronger repressor binding) until the branching point is reached. In the branching point repressor concentration continues to increase until a level is reached at which the population divides in into two strategies: Low receptor affinity and low virulence and high receptor affinity and high virulence. These strategies

are equivalent to lysogenic and ultra-virulent mutants of phage λ. (B) Accordingly, evolution decreases virulence until the branching point is reaches and disruptive selection leads to the co-existence of virulent and non-virulent population. (Parameters $\beta = 0.8$, $\gamma = 4$, $c_0 = k_0 = 0.5$*).*

Viral superinfection inhibition, as described in the case of phage λ has important consequences for the properties of the superinfection function and the evolution of virulence. First, in a host population which is fully infected by pathogen that deploys superinfection inhibition the relative benefit of horizontal transmission is reduced. Second, in phage λ, the molecular link between increased viral virulence and increased susceptibility to superinfection create a cost for virulence, since a more virulent virus has a higher probability to be displaced from its host cell by a superinfecting competitor. These two aspects of superinfection inhibition increase the relative benefits of vertical transmission and therefore enable the persistence of a fully vertical transmitting viral population or the co-existence of horizontal and vertical transmission strategies. The increase in the benefit of vertical transmission allows for the ecological specialization and co-existence of a non-virulent vertically transmitting 'defense' strategy and horizontally transmitting 'attack' strategy. Under these conditions a non-virulent virus can persist in the presence of a virulent counterpart.

The co-existence of virulence strategies has been described earlier. Two main properties of the superinfection function are decisive for the possible co-existence of virulence strategies. These are the slope of the superinfection function and the smoothness of the superinfection function, e.g. its behavior in the point of equal virulence of resident and superinfecting pathogen (Pugliese 2002; Boldin and Diekmann 2008). Most superinfection models assume a superinfection function that decreases with resident virulence and focus on the effect of non-smoothness around the point of equal virulence between resident and superinfecting pathogen. When the superinfection function has a strong non-smoothness in the origin, e.g. is a step function of virulence, a

large number of virulent strains can coexist around a virulence optimum (Nowak and May 1994). This is however an extreme case since a step-wise superinfection function enables mutants with infinitesimally small differences in virulence to enter and persist in the population (Pugliese 2002). A more realistic approach is the use of a piece-wise differentiable superinfection function $\phi_{RM}(\alpha_R, \alpha_M)$ that is $\phi_{RM} = 0$ zero for $\alpha_R > \alpha_M$ and steadily increasing for $\alpha_M > \alpha_R$. This less extreme non-smooth behavior of a piece-wise differentiable function can promote ecological and evolutionary and coexistence (Pugliese 2002; Boldin and Diekmann 2008).

The example of phage λ differs from these previous models in two important aspects. First, in contrast to previous models of superinfection the example of phage λ considers simultaneous horizontal *and* vertical transmission. Second, the mechanisms of superinfection inhibition introduce interference competition next to competition for host resources. Under these conditions the evolutionary coexistence of horizontal and vertical transmission strategies as well as the stable persistence of a fully vertically transmitting host population can occur even with a smooth superinfection function, under the premise that virulence and the susceptibility to superinfection show a positive (concave) relation.

Due to its stabilizing effect on vertical transmission strategies superinfection inhibition might play an important role in the maintenance of benignity in viral systems in general and therefore create an alternative explanation for the evolution towards lowered levels of virulence. Currently the major factor for the evolution of reduced virulence is seen in the ecological feedback of virulence on between-host transmission. Selection towards lowered virulence due to ecological feedback of virulence onto transmission might however be relatively weak in comparison to selection for increased virulence during within host competition during co-infection (Ebert and Bull 2003). In the absence of ecological feedback, other mechanisms are

required to enable the persistence of benign viruses. Superinfection inhibition might be an important mechanism for the maintenance of low virulence and should therefore be a common characteristic of benign viruses.

Indeed mechanisms that link viral virulence to superinfection inhibition which are similar to the mechanisms of phage λ can be pointed out in several other viral systems. Taking a closer look at other examples of benign DNA and RNA viruses, next to bacteriophage λ, we can see that mechanisms which relate of virulence to superinfection are indeed, common. Even though the genome organization of benign DNA and RNA can be vastly different these mechanisms show a certain 'core theme': Gene products that are involved in the limitation of viral self-replication are often used to suppress competing viruses. This regulatory core theme seems to have emerged multiple times. The single stranded DNA (ssDNA) phage M13, for example, causes a chronic infection of its host E.coli with a relatively mild effect on host mortality. In order to achieve this low level of virulence, M13 produces large amounts of protein P5 that on the one hand covers the single stranded form of M13 to prevent a conversion to the double stranded DNA (dsDNA) replicative form (RF) and on the other hand P5 inhibits the P2 gyrase that is required for the RF rolling circle replication. This way the replication repressor P5 limits the intra-host replication of M13 and, at the same time, blocks the replication initiation of a super-infecting M13 (Baas 1985). Therefore virulence and superinfection resistance are also tightly coupled in M13, although the mechanism is very different from virulence repression in phage λ. Another example is the retro-virus Hepatitis B (HBV), which causes a chronic liver infection. In order to escape immune suppression the HBV strictly limits its intra-cellular replication. It achieves this regulation by auto-repression of its reverse transcription protein P. This way reverse transcriptase P activity is high in the initial phase of infection but is repressed when protein P accumulates (Cao and Tavis 2006). When any competing HBV therefore enters an infected cell as a pre-genomic

RNA stage at a late stage of infection, its reverse transcription is also blocked by high levels of protein P that are produced by the residing virus, and superinfection is prevented. This way self-repression and superinfection inhibition are directly coupled in HBV.

Another class of retro-viruses, the Foamy Viruses use a system that is more similar to the one of phage λ. Foamy viruses cause benign infection. The genome of Foamy Virus contains next to the *gag*, *pol* and *env* proteins, the genes *tas* and *bet* that control the switch between latent, chronic viral infection and lytic viral replication. Thereby *tas* stimulates a switch to lytic replication, whereas *bet* represses the internal promoter and therefore suppresses a switch to the lytic stage. Strikingly, the expression of *bet* in Foamy Virus free cells has also been shown to provide resistance to superinfection by other Foamy Viruses (Nethe et al. 2005). The mechanisms of coupling between virulence and superinfection inhibition is therefore analogous to the cro and cI system in phage λ.

These examples suggests that genetic mechanisms that link virulence with increased susceptibility to superinfection, have evolved multiple times and could therefore be of major importance for the maintenance of viral benignity. The interpretation of the genetic coupling of increased virulence with increased susceptibility to superinfection might have analogies to the cost of 'cheater' strategies in kin selection theory that are required to maintain cooperation. Increased sensitivity to superinfection seems to be an intrinsic cost for high virulence. These associated costs of virulence are often required to stabilize the persistence of prudent host exploitation, i.e. low virulence against the invasion of virulent mutants (van Baalen and Sabelis 1995a; West et al. 2006). This way the genetic coupling of virulence and susceptibility to superinfection can be seen as a self applied limitation mechanism for the levels of virulence, which is 'hard-wired' in the viral gene regulation scheme. More attention should therefore be given to the evolution of mechanisms of viral superinfection inhibition as they have

the propensity to be at the core of the maintenance of low virulence in viruses.

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Chapter 6
Opposing selection pressures on receptor destroying enzymes of influenza virus limit viral adaptation and tissue specificity

Thomas W. Berngruber[1], Anke Huckriede[2], Franz J. Weissing[1],

1 Theoretical Biology Group, Centre for Ecology and Evolutionary Studies, University of Groningen, 2 Molecular Virology Group, University Medical Center, Groningen

6.1 Abstract:

Opposing selection pressures on a viral protein can impose severe limits on viral evolution. The receptor destroying enzyme neuraminidase of influenza virus is an intriguing example. During late viral development destruction of the viral target receptor is required to release newly formed viral particles from the host cell. Early in the viral life cycle, however, receptor destruction can abort viral infection in the stage of host cell attachment. It is therefore not straightforward to predict the evolution of neuraminidase activity in different host and

tissue environments. By means of a viral life history model we demonstrate that optimal viral adaptation to an environment requires specialization in the rate of receptor destruction that can prohibit viral replication in other hosts or tissues. Adaptation of the receptor destroying activity to local conditions can therefore provide a plausible explanation for viral host and tissue specificity and, as a consequence, differences in viral transmissibility and virulence.

6.2 Introduction

Viral reproduction requires the balancing of many genetic and biochemical processes acting at various stages in the viral life cycle. Evolutionary adjustment of this balance is often constrained by conflicting selection pressures that arise when a change that is advantageous during one stage of the viral life cycle is disadvantageous at another stage. In lytic bacterial viruses, for example, reduced lysis time is coupled to a reduction in viral burst size (Wang et al. 1996; Bull et al. 2004); and in polio virus there is a trade-off between the rates of viral genome replication and viral genome encapsidation (Krakauer & Komarova 2003). When two fitness determining components of the life cycle are linked through a trade-off the outcome of evolution cannot be predicted on the basis of each component independently. Instead, an evolutionary analysis requires the integration of fitness effects over the whole viral life cycle (Stearns 1992; Caswell 2001).

Receptor destroying enzymes are an important class of viral proteins that exemplify such opposing selection pressures. These enzymes play an important role in the life cycle of a large family of human and animal pathogens including *Ortho-* and *Paramyxoviridae* (influenza, para-influenza, Newcastle disease virus, mumps and measles) and *Corona-* and *Toroviridae* (Smits et al. 2005; de Groot 2006). A well-studied representative of these families is influenza virus. Influenza virus contains two major surface proteins: the receptor binding protein

hemagglutinin (HA) and the receptor destroying enzyme neuraminidase (NA). HA and NA are essential for viral reproduction and determine important viral properties like host specificity, tissue tropism, virulence and transmissibility (Baigent & McCauley 2003). Yet, the HA and NA proteins counteract each other at several points of the viral life cycle. Whereas the binding of HA to the host sialic acid (SA) receptors establishes viral attachment to the host cell, NA destroys these SA receptors and therefore potentially hampers virus-host attachment in the early viral life cycle. Receptor destruction therefore 'does not seem like a good idea', was it not that the receptor destroying activity of NA is indispensable in at least three other steps of the life cycle: NA activity prevents the aggregation of viral particles in the mucus layer of epithelia in the lung and the intestine (Matrosovich & Klenk 2003; Matrosovich et al. 2004), it enhances the passage of viral particles through the endosome of the host cell (Suzuki et al. 2005) and it prevents the accumulation of newly formed viral particles on the host cell surface after viral budding from infected cells (Palese et al. 1974). Nevertheless, due to its detrimental effect on virus-host attachment, the receptor destroying activity of NA remains a double edged sword and needs to be carefully balanced in accordance with the strength of HA binding and the properties of the available binding receptors (Mitnaul et al. 2000; Wagner et al. 2000; Wagner et al. 2002; Bin et al. 2005).

The environment of a virus is determined by the availability and properties of viral binding receptors in a specific tissue and host or tissue. Accordingly, host and tissue characteristics markedly affect the optimal balance of receptor binding and receptor destruction. The occurrence of cell mucus is one of these characteristics. Mucus contains decoy SA receptors that act as surrogate binding targets for viral HA and immobilize and inactivate viral particles. Liberation from mucus attachment requires the destruction of mucus SA receptors by viral NA activity (Matrosovich & Klenk 2003). In addition to the occurrence and density of mucus, the variation in the binding efficiency of SA receptors also plays an important role. Hosts and tissues differ, for

example, in their concentration and relative frequency of 2,3-α-gal-SA and 2,6-α-gal-SA receptors, and avian and mammalian influenza virus differs markedly in the affinity of HA and NA with both types of receptor (Gambaryan et al. 2006).

Due to the multiple effects of receptor destroying enzymes on the viral life cycle, the evolution of NA activity is a complex process. A quantitative understanding of the evolution of NA activity requires the integration of costs and benefits of receptor destruction in a life history model for the viral life cycle (Stearns 1992; Caswell 2001). Such a model describes the transitions between the various stages of the life cycle by a life cycle graph that corresponds to a system of differential equations. It is then straightforward to derive viral fitness from the properties of the stage transition matrix. Obviously, viral fitness reflects the properties of host and tissues. Hence, a life history models not only allows to determine the optimal level of activity of receptor destroying enzymes, but also the degree to which a virus with an enzyme that is adapted to one particular host or tissue can survive in a different host or tissue. Here we study the evolution of NA activity and HA avidity of influenza virus in such a life history model. We derive the NA activity and HA avidity that maximize the overall growth rate of the viral population. Our emphasis is on the host and tissue dependence of these viral properties and the question whether, and to what extent, local adaptation can provide and explanation for viral host and tissue specificity.

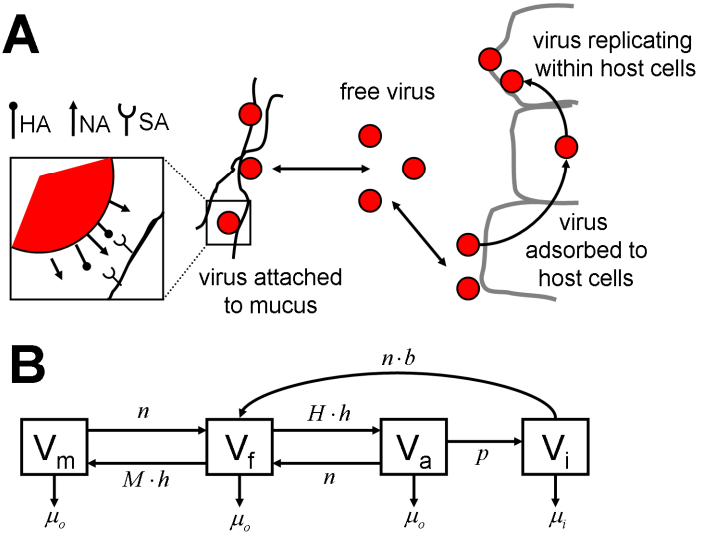

Figure1: The viral life cycle (A) Key processes in the life cycle of influenza virus. The virus can occur in four states: freely moving through the tissue, attached to mucus, attached to a host cell and internalized in a host cell, where it produces offspring virus. Viral attachment to mucus and host cells depends on the density and biochemical properties of sialic acid (SA) receptors and the avidity h of viral hemagglutinin (HA) to these receptors. Release from both, mucus and host cell attachment, to the free stage depends on the receptor destroying activity of the neuraminidase (NA) enzyme. (B) Life cycle graph summarizing the key assumptions of the life history model. The variables V_f, V_m, V_a and V_i denote the concentrations of virus in the four stages free, mucus adsorbed, host adsorbed and internalized. The transition rate from an absorbed to the free state is given by neuraminidase activity n. Adsorption rate to mucus and host cells is given by the product of hemagglutinin avidity h and the concentration H of mucus receptors and M of host cell receptors, respectively. Viruses adsorbed to a host cell are internalized with rate p and newly formed virus particles bud from infected cells at rate b and detach from the cell surface through neuraminidase activity n. Virus particles die at rate μ_o outside the host cell and at rate μ_i inside the host cell.

6.3 A model of HA-NA balance and its evolution

The structure of our model is motivated and summarized in Figure1. We consider four stages of the viral life cycle: free viral particles, mucus-adsorbed particles, particles that are adsorbed to the host cell, and particles that replicate within a host cell. The concentrations of the viral particles in these four stages are represented by the variables V_f, V_m, V_a and V_i, respectively. A virus from the pool of free particles can either enter the pool of mucus-adsorbed virus or host-cell adsorbed virus at a rate that is proportional to the receptor binding avidity h and the abundance of its preferred receptors in the mucus and on the host cell, which are denoted by M and H, respectively. Viral particles destroy receptors and detach from mucus and host cells at a rate determined by the receptor destroying activity n. Thereby the transition from the pool of host-adsorbed virus to free virus represents the abortion of infection, e.g. the negative side effect of receptor destruction. Viral particles that are adsorbed to the host cell penetrate the cell at rate p and enter the stage of replication within the host cell. Replicating particles in V_i produce new viral particles that bud from the host cell at rate b. Budded viral particles accumulate at the cell surface and require receptor destroying activity to detach and enter the pool of free viral particles, thereby finishing their life cycle. The rate of release from each infected cell is therefore proportional to the budding rate b and the detachment of viral particles n. All particles outside of the host cell decay at rate μ_o, while particles inside the host cell decay at rate μ_i. All these assumptions are represented by the life cycle graph in Figure 1B or, equivalently, by the following system of differential equations

$$\frac{dV_m}{dt} = -nV_m + hMV_f - \mu_o V_m \quad (2a)$$

$$\frac{dV_f}{dt} = +nV_m - hMV_f - hHV_f + nV_a + nbV_i - \mu_o V_f \quad (2b)$$

$$\frac{dV_a}{dt} = hHV_f - nV_a - pV_a - \mu_o V_a \quad (2c)$$

$$\frac{dV_i}{dt} = +pV_a - nbV_i - \mu_i V_i \quad (2d)$$

The properties of this linear system are determined by the 4 x 4 matrix of transition rates between states (Caswell 2001). In particular, the asymptotic growth rate λ of the viral population is given by the dominant eigenvalue of this matrix. Explicit calculation of λ, which we will later use as a measure of viral fitness, is intricate and inspires little insight. We therefore simplify the problem by making use of the different time scales of receptor binding and destruction and the process of viral replication. Receptor binding and destruction are spontaneous, fast, and energy-independent processes. In contrast, the processes of host cell penetration, replication within the cell and budding require complex interactions with the host cell and are therefore slow relative to receptor binding and destruction. Therefore, these two types of processes occur on different time scales. This means that on the fast time scale the particles redistribute very rapidly over the free, host-adsorbed and mucus-adsorbed stages, before the total number of viral particles noticeably changes due to the production of new particles. It is therefore reasonable to assume that the particles outside of the host cell reach a quasi-steady-state (Segel 1984) that is characterized by

$$\frac{dV_m}{dt} = \frac{dV_f}{dt} = \frac{dV_a}{dt} = 0 \ . \tag{3}$$

As a consequence the asymptotic growth rate λ of the viral population corresponds to the per capita rate of new infections, which according to (2d) is given by

$$\lambda = \frac{1}{V_i}\frac{dV_i}{dt} = p\frac{V_a}{V_i} - nb - \mu_i \tag{4}$$

In quasi-steady-state the viral particles distribute over the extra-cellular stages in the proportions

$$\tilde{V}_a = \frac{hH}{n + p + \mu_o}\tilde{V}_f = r_1\tilde{V}_f \tag{5a}$$

$$\tilde{V}_m = \frac{hM}{n + \mu_o}\tilde{V}_f = r_2\tilde{V}_f \tag{5b}$$

$$\tilde{V}_f = \frac{nbV_i}{h\left(M + H\right) - n\left(r_1 + r_2\right) + \mu_o} \tag{5c}$$

The ratios r_1 and r_2 represent the proportions of viral particles on the host cell and in the mucus relative to the amount of free virus

($r_1 = \tilde{V}_a / \tilde{V}_f$, $r_2 = \tilde{V}_m / \tilde{V}_f$). Substituting $\tilde{V}_a / V_i = r_1 \tilde{V}_f / V_i$ into (4), we get an explicit expression for the asymptotic growth rate of the virus:

$$\lambda = \frac{1}{V_i}\frac{dV_i}{dt} = \frac{p r_1 n b}{h(M+H) - n(r_1 + r_2) + \mu_0} - nb - \mu_i \qquad (6a)$$

In the appendix we show that under the assumption $\mu_0^{\,2} << H$ (that we will make from now on) this expression simplifies to

$$\lambda = nb\left[\frac{p}{p + \mu_0\left[1 + \frac{1}{H}\left(M + \frac{n+p}{h}\right)\right]} - 1\right] - \mu_i \qquad (6b)$$

The growth rate λ is a function of viral properties (n, h, p, b) and the conditions of the host tissue environment (M, H). We can therefore use λ to derive the optimal combination of the biochemical strategies, h and n, for a given host tissue that is characterized by M and H.

6.4 Optimal receptor destroying activity

The optimal receptor destroying activity n that maximizes the viral per-capita growth rate $\lambda(n,h)$ can be found by calculating the maximum of $\lambda(n,h)$ in the direction of n.

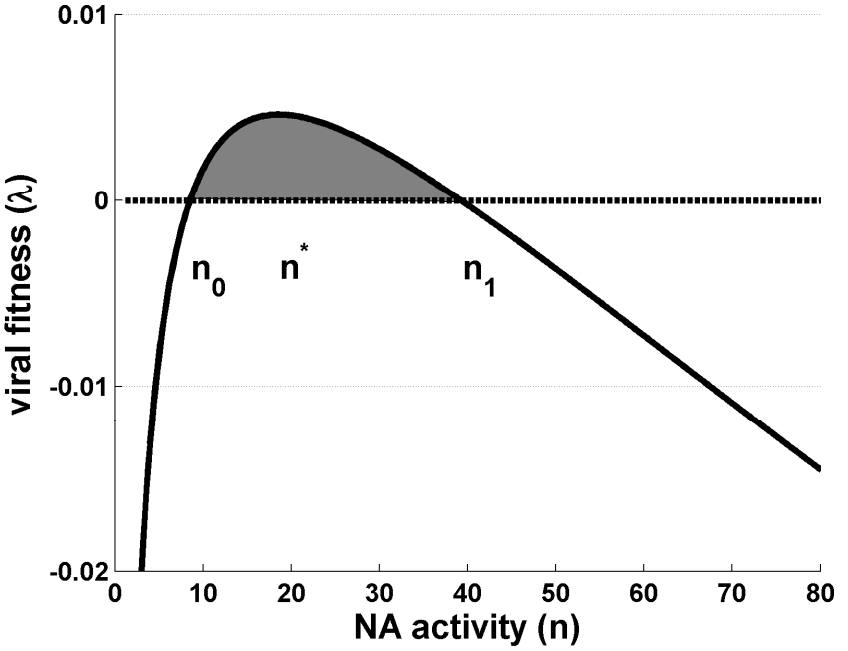

Figure 2: Viral fitness as a function of neuraminidase activity. A virus with a low NA activity $n \approx 0$ tends to accumulate within the host. Therefore it has a negative growth rate that is approximately given by $\lambda = -\mu_i$. A virus with a high NA activity tends to accumulate in the free state. Accordingly, it also has a negative growth rate that is approximated by $\lambda = -\mu_o$. Some host tissues do not allow viral growth irrespectively of the value of n. In the example presented here, there is a tissue specific interval $n_0 < n^* < n_1$ allowing viral growth. Viral fitness (= asymptotic growth rate) is maximized at an intermediate neuraminidase activity n^* (Parameter values: $h = 1$, $b = 3$, $p = 10^{-2}$, $\mu_o = \mu_i = 0.15$, $M = 1$, $H = 1$).

This optimum n^* is given by

$$\left.\frac{\partial\lambda(n,h)}{\partial n}\right|_{n=n^*} = 0 \qquad \text{and} \qquad \left.\frac{\partial^2\lambda(n,h)}{\partial n^2}\right|_{n=n^*} < 0\,. \qquad (7)$$

Let us first try to understand intuitively the effect of receptor destroying activity n on viral growth rate. It is easy to see that neither a very small n nor a very large n are beneficial. For $n=0$, all viral particles accumulate in infected hosts, where they decay at rate μ_i. Therefore the growth rate $\lambda=-\mu_i$ is negative for small n, implying viral extinction. On the other end, for large n, the ratios r_1 and r_2 become small and all viral particles accumulate in the free stage where they decay at rate μ_o. Large n therefore also leads to a negative growth rate $\lambda=-\mu_o$ and, accordingly, also to extinction. It is possible that the host tissue is too hostile to allow viral growth: $\lambda(n,h)<0$ for all n. For other tissues, however, λ is positive for intermediate values of n, i.e. for values from an interval of $n_0<n<n_1$, where n_0 and n_1 reflect the tissue properties M and H and the receptor binding avidity h (Figure 2). As shown in the appendix, the optimal receptor destroying activity of n^* is given by

$$n^* = \frac{h}{\mu_o}\left(\sqrt{pHu}-u\right) \qquad \text{with} \qquad u = pH+\mu_o\left(H+M+\frac{p}{h}\right) \qquad (8)$$

We can conclude that viral growth is negative for extreme values of n and optimal for an intermediate level of receptor destroying activity n^*.

The optimum n^* depends on the receptor binding avidity h and the properties of the target tissue H and M. For this reason differences in H and M between target tissues will lead to different optima in the receptor destroying activity n^* and different regions of positive viral growth $n_0 < n < n_1$. In the next section we will demonstrate how these differences in optimal receptor destroying activity n^* can prevent viral spread between tissues of different receptor availability H and M.

In our model the situation is markedly different for HA avidity h. As shown in the appendix, the asymptotic growth rate of the virus is positively related to the rate of receptor binding avidity h. Therefore h should always evolve towards the maximal attainable value.

6.5 Differences in optimal rates of receptor destruction determine tissue specificity

In order to determine if a virus can spread between different tissues we need to determine whether a virus that is adapted to a tissue of origin X has a positive growth rate in a certain target tissue Y. Within one host organism tissues differ in their relative abundance of receptors in the mucus M and on the host cell H. A virus that is adapted to a tissue X with an optimal strategy n_X^* will be able to spread to a target tissue Y when the growth rate $\lambda\left(n_X^*\right)$ of a virus with NA activity n_X^* is positive in tissue Y. The target tissue Y in turn only provides a positive viral growth-rate for a receptor destroying activity n within the interval $n_{0,Y} < n < n_{1,Y}$. A virus can therefore not spread from tissue X to Y when n_X^* falls outside the interval $n_{0,Y} < n < n_{1,Y}$ (see Figure 3).

Lets now consider which viruses that are adapted to a different environment can invade a given reference environment. To calculate whether a virus from an environment $X = \left(H, M\right)$ can invade the

reference environment Y we calculated the optimal strategy n_X^* and determined whether $n_{0,Y} < n_X^* < n_{1,Y}$. This procedure divides the parameter space into environments X that do not allow viral growth at all [i.e. $\lambda\left(n_X^*\right) < 0$; black region in Figure 4], regions that allow growth in the reference environment Y [i.e. $n_{0,Y} < n_X^* < n_{1,Y}$; white region in Figure 4] and in regions where viruses adapted X cannot grow in Y [gray regions in Figure 4]. For a given value of M viral growth does not occur below a threshold level for the concentration of host receptors H. This threshold increases with M. The minimal level of H that is required for viral growth can be approximated by a linear function of M (see Appendix). Combinations of M and H that allow for viral growth, or $\lambda\left(n_X^*\right) > 0$, fall into two categories: Environments which produce viruses that can invade the environment Y and those which cannot. Intuitively, environments X which are more similar to environment Y can produce viruses that can spread from X to Y. Distance in the direction of M and H has, however, a different effect on tissue specificity. Whereas viruses from an environment with a higher value of H can all invade the reference environment Y, viruses from environment of either very low or very high values of M are not able to invade the reference environment. Accordingly, the mucus concentration M is the primary factor that causes tissue specificity (Figure 4).

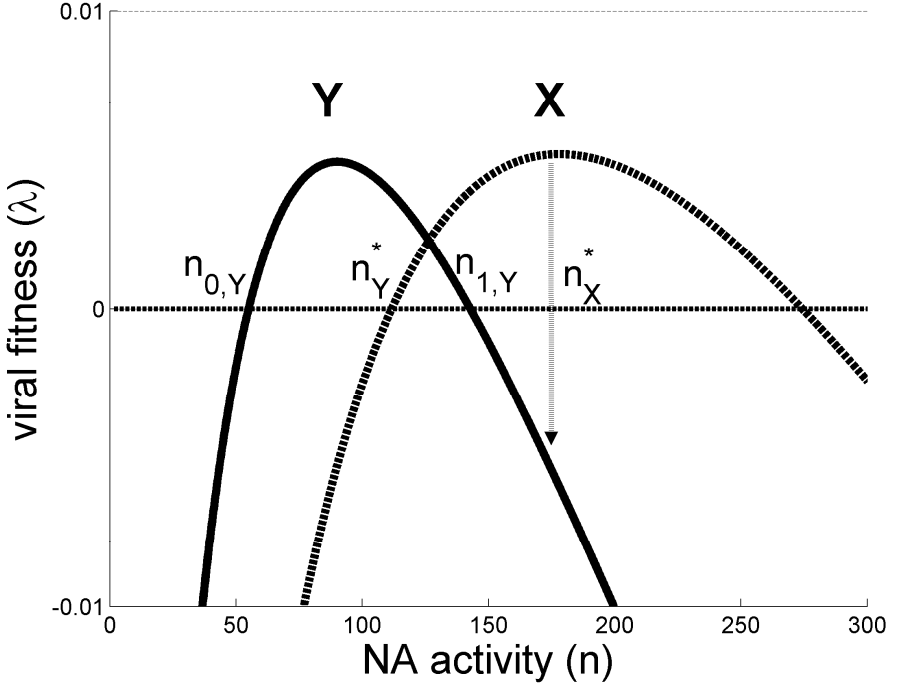

Figure 3: Tissue specificity of viral adaptation. Dependence of viral fitness (=asymptotic growth rate) on neuraminidase activity for two tissues X and Y. The tissues differ in the concentration of SA receptors on the host cell ($H_X = 3.7$, $H_Y = 2$) and in the surrounding mucus ($M_X = 200$, $M_Y = 50$). In environment Y, viral growth is possible for n-values between $n_{0,Y}$ and $n_{1,Y}$. The virus optimally adapted to environment X, does not satisfy this requirement (since $n_X^* > n_{1,Y}$) and hence has a negative growth rate in environment Y (see dotter arrow). Other parameters: $h = 1$, $b = 3$, $p = 10^{-2}$, $\mu_o = \mu_i = 0.15$.

6.6 Discussion

Conflicting selection forces can severely hamper viral adaptation. Here we studied the example of the receptor destroying enzyme neuraminidase (NA) of influenza virus to establish a framework that integrates conflicting enzymatic effects into the viral-life cycle. We furthermore use this model to predict viral adaptation to the environment. Our model demonstrates that viral adaptation to the prevailing tissue environment requires specialization of receptor destroying activity that can prohibit spread of the virus to other tissues. This specialization in receptor destroying activity could provide an additional mechanism for viral tissue and host specificity, next to other mechanisms, like the presence of suitable receptors and necessary proteases.

Viral fitness in the situation of an *in vivo* infection is affected by multiple factors like the viral replication rate, the ability to avoid the immune system and the rate of transmission between host organisms. In our model we focus on a single aspect of viral fitness which is its replication rate. Viral replication rate is generally strongly related to fitness. Maximal fitness can, however, deviate from the maximal replication rate, when viral replication has a negative side effect on other components of viral fitness. Viral replication can for example increase host mortality and therefore negatively affect viral transmission (Ewald 1994; van Baalen & Sabelis 1995); but see also (Ebert & Bull 2003). Furthermore, viral replication can have negative consequences for the avoidance of the specific immune system. Rapidly replicating viral mutants, for example, create a higher antigen dose and therefore a stronger specific immune response. In principle, this coupling between replication, antigen dose and immune response can select against strains with highest replication rate and create a rareness advantage of slow replicating mutants (Nowak et al. 1991). In some cases replication rate and immune avoidance can also be determined by a single mutation (Both et al. 1983). In our model, we do not address the action of a specific immune response. Instead, we

represent a non-specific immune response in terms of the decay parameters μ_o and μ_i. In principle the negative side effects of viral

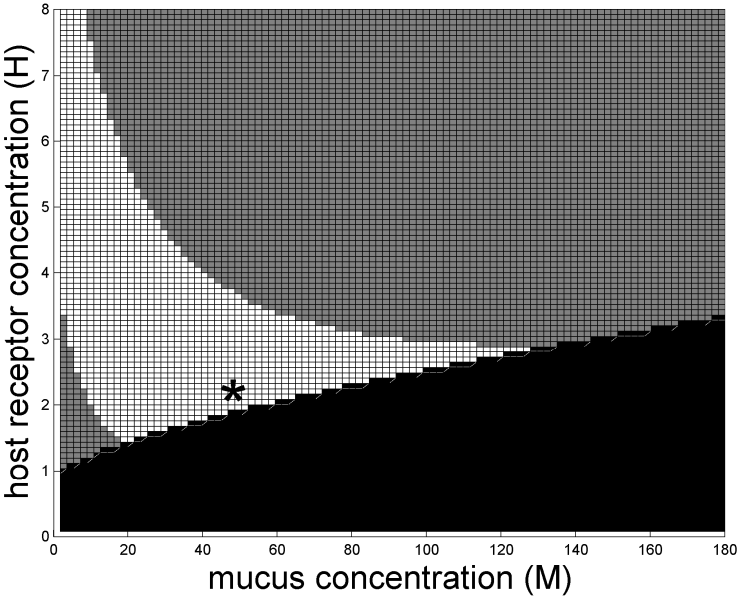

Figure 4: Parameter dependence of viral extinction and tissue specificity. Viral growth strongly depends on the tissue characteristics H (= concentration of SA receptors on host cells) and M (= receptor concentration on mucus). The black region corresponds to those tissues where viral growth is impossible irrespective of the value of neuraminidase activity n. The star marks a specific reference environment characterized by $M = 50$ and $H = 2$. The white region corresponds to those tissues where the virus optimally adapted to the given tissue is able to grow in the reference environment. Viruses derived from tissues in the grey parameter region cannot grow in the reference environment. Other parameters: $h = 1$, $b = 3$, $p = 10^{-2}$, $\mu_o = \mu_i = 0.15$.

replication onto future transmission and on immune avoidance can create other interesting trade-offs in addition to the here described conflicting selection on receptor destroying activity, which are

addressed elsewhere (Both et al. 1983; Nowak et al. 1991; Ewald 1994; van Baalen & Sabelis 1995). Assuming that the specific immune system is not affecting the early stages of viral infection we can nevertheless use the maximization of replication as a predictor for viral fitness as has been done by previous models for within-host viral growth [e.g. (Regoes et al. 2005)].

The intermediate optimum for receptor destroying activity has profound consequences for viral evolution. Most importantly, the adaptation to specific receptor destroying activity can prevent the spread of virus between different hosts and tissues. Yet, one has to consider the role of different optima in receptor destroying enzymes in relation to other mechanisms that determine viral tissue tropism. Viral spread between tissues is, for example also affected by the availability of cellular proteases that cleave the HA precursor HA0 to the active form of HA. For seasonal human influenza viruses and low pathogenic avian viruses the required proteases are expressed tissue-specifically. In contrast, highly pathogenic viruses, of avian origin (HPAI), contain a multibasic cleavage site which allows cleavage of HA_0 to its active form by ubiquitous proteases. Therefore, replication of HPAI does not require the presence of proteases that are specific for lung tissues. Consequently, avian HA_0 can be cleaved in tissues outside the lung which enables unrestricted viral spread and systemic infection. In avian hosts, the occurrence of a multibasic cleavage site shows a strong correlation with tissue tropism and virulence (Horimoto & Kawaoka 1994). In contrast, in mammalian hosts this correlation is weaker (Steinhauer 1999). In mammalian hosts, tissue differences in optimal receptor destroying activity could therefore be an important factor for tissue specificity, next to HA cleavability. Ultimately, the role of different optima of receptor destroying activity for tissue specificity remains, however, an empirical question.

Inhibitors of the receptor destroying activity of NA are currently the only possible pharmaceutical intervention for an influenza infection.

Obviously, NA inhibiting drugs disturb the balance of viral attachment and receptor destruction and therefore inhibit viral replication. Adaptation to NA-inhibitors can, however, readily occur through restoration of the attachment/detachment balance (Gubareva et al. 2001; Gubareva 2004; Reece 2007). Quantitative understanding of the evolutionary limitations of receptor destroying enzymes is an important tool to understand the limitations of viral adaptation to NA inhibitors. For example, different optima for receptor destroying activity between tissues predict situations in which the application of inhibitors of receptor destroying enzymes (NA-inhibitors) actually can promote the spread of virus between tissues. This undesired effect of NA-inhibitors can occur when a virus, that is adapted to an environment with a high optimum of receptor destroying activity, enters a tissue or host organism that requires a low rate of receptor destroying activity. In this case, reduction of NA activity by NA inhibitors moves the receptor destroying activity closer to the optimal level in the new environment and increase viral replication. Even worse, when the difference of optimal receptor destroying activity prevents viral spread between tissues, NA inhibitors could facilitate spread to new tissues and promote systemic infection. NA inhibitors have not yet been shown to increase viral replication directly. However, mutations that decrease the receptor destroying activity can indeed increase viral replication (Bin et al. 2005). It is therefore plausible that reduced receptor destroying activity, can increase viral replication in tissues with a low optimal receptor destroying activity and facilitate the spread from mucus to non-mucus tissues, regardless whether receptor destroying activity is reduced by NA-inhibition or directed mutations.

Tissue specific optima of receptor destroying activity imply that the adaptation to one environment worsens the success in another environment. When viral tissue specificity affects virulence and transmission, this effect can create a trade-off between viral virulence and transmission. As mucus-adapted viruses will be more successful in the mucus tissues which form the entrance route of infection, mucus

adaptation should promote transmission between host organisms. The virulence of a viral strain in turn depends on its ability to spread to mucus free tissues within one host orgamism, causing systemic infection. Even though viruses that are adapted to mucus tissues should transmit readily between host individuals, they are maladapted for growth in mucus free tissues and should therefore show low virulence. In turn, viruses that are adapted to mucus free tissues should be virulent but poorly transmitting. There is some evidence that highly virulent viral strains, indeed, transmit poorly in experimental transmission experiment even when the contact rate of animals is very high (Yen et al. 2007).

The trade-off between the specialization onto mucus containing and mucus free tissues, respectively, poses the question whether a generalist virus can evolve, which is able to reproduce in both types of environments and is therefore highly virulent and transmittable at the same time. Considering the evolutionary limitations on receptor destroying activity the occurrence of such a highly transmissible and highly virulent mutant might be less likely then previously thought. Evaluation of this possibility should focus on the measurement of HA avidity and NA activity as quantitative kinetic parameters in various tissues and host species.

The examples above demonstrate that biochemical constraints between different parts of the viral life cycle can be valuable to illuminate the limitations of viral evolution. Although biochemical conflicts in viral adaptation are likely to be ubiquitous, models that derive evolutionary limitations directly from the underlying biochemistry are scarce, probably because they require virus specific models. Current biochemical models of the viral life cycle focus on generic processes of the viral replication, like the dynamics of viral genome replication and protein production. For example, Krakauer & Komarova (2003) have focused on within cell processes like the encapsidation of viral genomes and its effect on viral genome replication. Regoes and

colleagues (2005) have investigated the optimal ratio of positive and negative RNA strands that maximizes the replication of polio virus. The example of receptor destroying enzymes demonstrates that mechanisms for conflicting selection lure also in other parts of the viral life cycle besides genome replication and packaging, like the attachment and detachment processes. Even though the mechanisms that constrain the evolution of receptor destroying activity are rather specific, these constraints have implications for a large group of animal pathogens.

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6.8 Appendix

6.8.1 Existence of a region of positive viral growth

In view of (6) and the definition of r_1 and r_2 in (5) the asymptotic growth rate of the virus is given by

$$\lambda = \frac{1}{V_i}\frac{dV_i}{dt} = nb\left[\frac{phH}{\left(n+p+\mu_0\right)\left[h\left(M+H\right)-n\left(\frac{hH}{n+p+\mu_o}+\frac{hM}{n+\mu_o}\right)+\mu_o\right]}-1\right]-\mu_i \tag{A1}$$

This can be rewritten as

$$\lambda = nb\left[\frac{p}{p+\mu_0+\frac{\mu_0}{H}\left[M+\frac{n+p}{h}\right]+\frac{\mu_0^2}{H}\left[\frac{M}{n+p}+\frac{1}{h}\right]}-1\right]-\mu_i \qquad \text{(A2)}$$

For large n the quotient in (A2) converges to zero. Accordingly, the term in brackets, and with it the per capita growth rate λ, becomes negative for large values of n and converges to $\lambda = -\mu_o$. On the other end $\lambda = -\mu_i$ for $n = 0$. Therefore there are two scenarios: either λ is negative for *all* values of *n* (implying that the virus cannot persist), or λ is positive for an interval $n_0 < n < n_1$. An optimal n^* that supports viral growth, exists in this interval, when $\lambda(n)$ is concave and $\lambda(n^*) > 0$. To simplify the analysis we assume that ${\mu_0}^2 << H$ allowing us to drop the small term $\frac{\mu_0^2}{H}\left[\frac{M}{n+p}+\frac{1}{h}\right]$ from (A2) to arrive at

$$\lambda = nb\left[\frac{p}{p+\mu_0\left[1+\frac{1}{H}\left(M+\frac{n+p}{h}\right)\right]}-1\right]-\mu_i \qquad \text{(A3)}$$

It is now easy to see that λ is positive when

$$\frac{1}{H}\left(M+\frac{n+p}{h}\right) > x_0 = \frac{p}{\mu_0}\left[\frac{nb}{\mu_i+1}-1\right]-1 \qquad \text{(A4)}$$

or, equivalently

$$H > \frac{1}{x_0}\left(M+\frac{n+p}{h}\right) \qquad \text{(A5)}$$

Correspondingly λ is positive when

$$h > \frac{n+p}{x_0 H - M} \qquad \text{(A6)}$$

We can conclude from (A5) that for $\mu_0^{\,2} << H$ the minimal H permitting viral growth is approximately linearly related to M (see also Figure 4A) and from (A6) we can see that the minimal h that enables viral growth asymptotically approaches infinity for $M \to x_0 H$ (see Figure 4B). Therefore at the critical mucus concentration $M_c = x_0 H$ no further increase in h can support viral growth.

6.8.2 Optimal NA activity and HA avidity

In order to calculate the optimal NA activity n^* we solve the equation $\partial\lambda / \partial n = 0$ which yields

$$n^* = \frac{h}{\mu_0}\left(\sqrt{pHu} - u\right) \quad \text{with} \quad u = pH + \mu_0\left(H + M + \frac{p}{h}\right) \tag{A7}$$

Furthermore, n^* is a local maximum when λ is concave in n or

$$\frac{\partial^2 \lambda}{\partial n^2} < 0 \qquad \Leftrightarrow \qquad \mu_0\left(H + M + p\right) + hpH > 0 \tag{A8}$$

This implies that n^* is always a local maximum for positive values of μ_0, h, p, H, M.

In contrast to the selection for intermediate for NA activity n^*, HA avidity h should evolve to its attainable maximum. This can be seen directly from (A3). When $h \to \infty$ the viral growth rate $\lambda(h)$ increases until it asymptotically reaches a maximum

$$\lambda_{\max} = nb\left[\frac{pH}{p + \mu_0\left(1 + \frac{M}{H}\right)} - 1\right] - \mu_i \tag{A9}$$

For high values of h the asymptotic growth rate is therefore insensitive to changes in h.

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Nederlandse samenvatting

Het reproductief succes van een virus is afhankelijk van een zorgvuldige balans tussen de temporele en kwantitatieve organisatie van reproductie in de levenscyclus van het virus. Deze balans wordt gewaarborgd door regulatie van de virale eiwitproductie en eiwitinteracties. De evolutie van virussen is afhankelijk van de mogelijkheden om deze eiwitinteracties optimaal op elkaar af te stemmen. In veel virale systemen zijn deze mogelijkheden sterk beperkt, vooral in situaties waarin het positieve effect van een eiwit inherent gekoppeld is aan nadelige neveneffecten – een zogenaamde moleculaire tradeoff.

Het doel van dit proefschrift is het identificeren van dergelijke moleculaire tradeoffs, het voorspellen van hun effect op de evolutie van virussen en het testen van deze voorspellingen in evolutionaire experimenten. Het speerpunt van onze aanpak is het reduceren van de biochemische complexiteit van een virus tot een gesimplificeerd model van de levenscyclus, om daarmee de cruciale biochemische stappen te identificeren die bepalend zijn voor de fitness van het virus. Door middel van deze aanpak worden de evolutionaire “zwaktepunten” van een virus geïdentificeerd. Kennis van deze evolutionaire zwaktepunten vormt een belangrijke basis voor de ontwikkeling van toekomstige antivirale strategieën die robuust zijn tegen virale adaptatie en resistentie.

De omgeving van een virus bepaalt de optimale afstemming van de eigenschappen van de levenscyclus van een virus. De optimale virulentie van een virus is bijvoorbeeld gerelateerd aan de dichtheid van de gastheer. In een lage gastheerdichtheid is het voor een virus nadelig om zijn gastheer teveel schade toe te brengen aangezien dit de

gastheer kan doden voordat het virus zich kan verspreiden naar een nieuwe gastheer. Theoretische modellen voorspellen daarom dat een virus zijn virulentie zou moeten aanpassen aan de gastheerdichtheid in zijn omgeving.

Aanpassingen van de levenscyclus van een virus zijn echter afhankelijk van de moleculaire mogelijkheden om deze levenscyclus te veranderen. Deze moleculaire mogelijkheden vormen de bron voor de mutaties die aanpassing aan de omgeving mogelijk maken. Beperkingen in deze moleculaire mechanismen zijn daarom van directe invloed op de aanpassing van een virus aan zijn omgeving. In theoretische modellen worden deze moleculaire mechanismen echter vaak buiten beschouwing gelaten. De introductie van expliciete moleculaire mechanismen in modellen van virale evolutie zou tot volledig nieuwe inzichten kunnen leiden.

In dit proefschrift ontwikkel ik methoden om moleculaire tradeoffs in de virale levenscyclus te identificeren, hun evolutionaire rol met behulp van wiskundige modellen te voorspellen, en deze voorspellingen door middel van experimentele evolutie van bacteriële virussen, zogenoemde bacteriofagen, te testen. Met deze aanpak laat ik in een viertal voorbeelden zien hoe moleculaire mechanismen de aanpassing van een virus aan zijn omgeving sterk kunnen beperken.

In hoofdstuk 2 onderzoek ik de consequenties van verstoorde gen expressie voor de fitness en adaptatie van het RNA virus MS2. Voor dit experiment werden de vier genen van MS2 één voor één in een productieplasmide gecloneerd. Met dit plasmide kunnen virale genen constitutief worden geproduceerd. Deze productie kan niet gecontroleerd worden door het virus en verstoort daardoor de natuurlijke balans van virale eiwitten tijdens de infectiecyclus. Dit heeft een sterk remmend effect op de groei van het virus, ook al produceert het plasmide viraal genproduct zonder dat het virus er “moeite voor hoeft te doen.” Het virus kan zich aan deze verstoring maar in beperkte mate aanpassen. Vooral de aanpassing aan een te hoog niveau van het

virale RNA replicatie enzym is lastig voor het virus. Overproductie van RNA replicatie enzymen blijkt zo even efficiënt als antivirale strategie, als de repressie van de productie van deze eiwitten – het doel van vele bestaande antivirale strategieën. Echter blijkt het voor het virus lastiger zich aan te passen aan de over-productie van RNA replicatie enzymen dan aan hun repressie. Dit biedt een perspectief voor een mogelijke antivirale strategie die bestand is tegen virale adaptatie.

Hoofdstuk 3 beschrijft de evolutie van de infectiviteit van bacteriofagen in enerzijds een vloeibare omgeving en anderzijds een ruimtelijk gestructureerde omgeving. Theoretische modellen voorspellen dat een te hoge mate van infectiviteit in een ruimtelijk gestructureerde omgeving nadelig is, omdat geïnfecteerde gastheercellen een barrière voor de verdere verspreiding van het virus vormen. Daarom kan een lage infectiviteit in een ruimtelijk gestructureerde omgeving voordelen hebben. In een vloeibare omgeving verdwijnen deze voordelen en zal een virus tot zijn maximale infectiviteit evolueren. Wij demonstreren dit effect door middel van experimentele evolutie van bacteriofaag ΦX174 in een ruimtelijk gestructureerde evenals in een vloeibare omgeving. Hoewel onze resultaten de theoretische verwachtingen bevesigen, hebben verdere controle-experimenten aangetoond dat deze resultaten beïnvloed zijn door de aanwezigheid van een tot nu toe onbekende (pro)faag in het genoom van de gastheerbacterie. De ontdekking van deze onbekende faag en zijn potentiële invloed op de experimentele evolutie van bacteriofaag ΦX174 is het onderwerp van hoofdstuk 4. Uiteindelijk identificeren wij in hoofdstuk 4 de onbekende faag door middel van sequentieanalyse als een virus uit de familie van lysogene virussen. Lysogene fagen kunnen zich via integratie in het gastheergenoom van moedercel tot dochtercel verspreiden. Deze manier van verspreiding wordt verticale verspreiding genoemd. Het bestuderen van deze vorm van virale verspreiding was de aanleiding voor het theoretische werk in hoofdstuk 5.

Hoofdstuk 5 beschrijft de evolutie van een virus dat zowel horizontale transmissie (tussen ongerelateerde gastheren) als ook verticale transmissie (van moeder tot dochtercel) vertoont. Een belangrijke voorwaarde voor verticale transmissie is het overleven van de gastheercel. Om overleven van de gastheercel te waarborgen moet een virus enerzijds zijn eigen virulentie beperken, maar anderzijds zijn gastheercel tegen de overname door een virulente virus-mutant beschermen. De mechanismen voor de beperking van virulentie en de bescherming tegen overname zijn in lysogene virussen nauw gerelateerd. Een virus dat zijn eigen virulentie beperkt is daarom automatisch immuun tegen overname door een virulent virus. Een virulent virus is daarentegen niet efficiënt in de bescherming van zijn gastheercel. Deze tradeoff leidt tot de mogelijkheid van coëxistentie van virulente (slecht beschermende virussen) en niet-virulente (goed beschermende virussen). Dit verklaart het voorbestaan van niet-virulente virussen in de aanwezigheid van virulente mutanten.

Hoofdstuk 6 beschrijft de tegenstrijdige effecten van het eiwit neuraminidase op de levenscyclus van het influenza virus en de gevolgen voor virale evolutie. Neuraminidase verbreekt de binding van het virus aan de gastheercel en heeft daarmee een nadelig effect op de vroege stadia in de virale levenscyclus. Anderzijds is de activiteit van neuraminidase noodzakelijk aan het einde van de virale levenscyclus wanneer nieuw gevormde virusdeeltjes de gastheercel verlaten. Neuraminidase heeft daarom tegelijkertijd voordelige en nadelige effecten op virale reproductie. De balans tussen deze relatieve voor- en nadelen is afhankelijk van de weefsels waarin een virus zich bevindt, en de aanwezigheid en kwaliteit van geschikte bindingsreceptoren. De optimale activiteit van neuraminidase verschilt tussen deze weefsels. Aanpassing van de neuraminidase-activiteit aan een bepaald type weefsel kan daarom de aanpassing aan een ander type weefsel verminderen. Een virus dat bijvoorbeeld aangepast is aan longweefsel heeft daardoor een neuraminidase-activiteit die groei op andere weefsels kan verhinderen. Aanpassing van het virus kan daarom tot

specialisatie voor een bepaald type weefsel leiden. Specialisatie in een bepaald type gastheerweefsel is op haar beurt een belangrijke factor voor virulentie en transmissie van het virus. De specialisatie van neuraminidase activiteit voor bepaalde typen weefsel is daarom een mogelijke verklaring voor de dichotomie tussen influenzastammen met hoge virulentie maar slechte transmissie en influenzastammen met lage virulentie maar goede transmissie.

Met deze vier voorbeelden wordt de rol van moleculaire beperkingen voor de optimale aanpassing van de virale levenscyclus aan de omgeving van een virus aangetoond. Onze aanpak vormt een algemene strategie om de evolutionaire zwaktepunten van specifieke virussen te identificeren en wijst de weg naar de ontwikkeling van antivirale strategieën die bestand zijn tegen de evolutie van virale resistentie.